Laboratory Diagnosis

Introduction, General Approach to Laboratory Diagnosis

You get a blood culture, a urine culture, or a culture of that pus pocket in the person's thigh. You order a Gram stain and culture. You wait two days for the culture to grow a bacterium and be reported in the computer, where you see the name of the bug and all the drugs and sensitivities. You choose one with an "S" (S for sensitive). Phew! Clinical micro is hard! That's what a hospitalist does when they admit someone for an infection. A basic science education does not require you be the microbiologist actually applying the stains and plating bacteria on growth media. But what it does require is that if you suspect an infection by a certain bug you must sometimes know to order a special test.

You provide the microbiology lab with a **specimen**. You order the Gram stain on part of that sample. You order culture and sensitivities on another. The lab saves a huge chunk of the sample just in case you want to run some special tests on it.

You ordered a **Gram stain**. Crystal violet, detergent, red counterstain, as described in Bacteria # 1: *Bacterial Structure and Introduction*. Gram-positive organisms are purple, gram-negative organisms are pink. OR if you didn't ask for a special stain and needed one, you would get a **negative Gram stain**. That's why we cover special stains in this lesson. Antibiotics are started empirically based on the diagnosis, and are usually not altered by the Gram stain alone. But if you don't know to order the special stain, the lab won't do it. A negative Gram stain doesn't mean there aren't organisms, it means either that there are no bugs OR the bugs that are there didn't take the purple or the red dye (because you missed what you needed to do).

You ordered a **culture**. If you don't specify anything, the sample is smeared onto a **blood agar plate** and the bacteria are incubated **in oxygen**. This culture is grown to do antibiotic susceptibility testing, visual diagnosis, and any special tests. If the bug you cultured was an anaerobe, and the lab grows the bacteria in oxygen, your bacteria probably won't grow. When blood cultures are ordered, both aerobic and anaerobic bottles are inoculated. If you don't specify up front, you get only aerobic. Specific agars are needed for some organisms. You should be aware of which plates to pick, which agars are needed for the growth of a bacterium, even if you aren't the one smearing it onto the agar yourself.

Your culture comes with **antibiotic susceptibility testing**. That culture starts to grow. The lab takes a piece of that happy growing culture and replates them to grow some more. Only this time, they are incubated with little antibiotic pellets on the agar. Any bacteria that grow next to an antibiotic pellet are resistant to that antibiotic. Where the bacteria don't grow, they are susceptible.

Sometimes, you need **special tests**. The culture does more than give you a pile of organisms to look at. It gives you a rich sample to perform additional tests (sugar fermentation, DNA probes, antibody agglutination, immunofluorescence, etc.) to ensure that you have the right species. A not rare but not uncommon thing to have happen in practice is to have a Gram stain say one thing, the initial culture say something else, and a final culture on day 5 updated to reflect the species name. Everyone knew it was gram positive and probably staph or strep on day of admission. We knew it was susceptible to TMP/SMX and thought it was *Staph. aureus* on day 2. On day 5 we realized that it was staph, was susceptible to TMP/SMX, but really it was actually *Staph. saprophyticus*.

In clinical practice the microbiology lab handles this for you. The micro tech does the laboratory algorithm for diagnosis, plates bacteria on different media, runs all the diagnostics, and then assembles the combined results of every test. A microbiologist looks at the result of all the tests and matches it to a reference sheet. All the inputs from all the tests run result in one bacterium's fitting the description. The only way we know that this colony is *E. coli* is because this colony's results of the lab's tests matches the standardized result of the lab's test that indicates *E. coli.* Genetic diagnosis and serotyping can be done in addition to the laboratory algorithm, but often are not.

And so learners think they must memorize that reference sheet, memorizing every microbiologic feature for every bacterium. Do not do that. Instead, use this lesson to be informed, oriented to the many tests that are performed. Then cluster. Which test is useful at what time point. To illustrate this concept, that reference sheet has susceptibility or resistance to novobiocin for every bacterium known. The only time we need to call on novobiocin is to differentiate *Staph. saprophyticus* from *Staph. epidermidis*. Since it doesn't matter whether any other bacteria are susceptible or resistant to novobiocin, you do not commit that data point to memory for any other bacteria other than these two. AND, only call on novobiocin when you have non-*Staph. aureus* staph species.

Like the preceding lessons, this lesson is a reference sheet, an orientation. You will refer back here as we move forward discussing the individual organisms. When we teach the bacteria and the diseases they cause, we will highlight which tests you need to memorize and leave off all the ones you don't.

Gram Stain: Color and Shape

Gram positives are purple. Crystal violet stains them purple and that stain is not removed by the lipid detergent. **Gram negatives are pink**. Crystal violet stains them purple, then their violet outer membrane is pulled off by the lipid detergent, and then the red counterstain stains them pink.

Cocci are circles. Bacilli are **rods**.

We'll show you how to use this information in the next lesson.

Properties of Growth Media

Selective. Selective media favor the growth of the particular organism for which they are selective AND **prevent growth of other organisms**. When you plate the bacteria, you don't yet know the microbe. But if you think you know from the clinical syndrome and Gram stain, and then choose a selective medium, you will confirm the diagnosis because the medium is selective for the organisms you guessed. An example of this is the **Thayer-Martin** agar. It contains antibiotics that DON'T work on *Neisseria*. *Neisseria* grows happily while any other bacterium is murdered by the antibiotics. If you think its *Neisseria meningitidis*, get the Thayer-Martin agar. If you have a meningitis you don't always get the Thayer-Martin agar.

Differential. Differential media, also called indicator media, are compounds that allow on species of bacteria to be distinguished from another based on a biochemical reaction. The classic example is detecting **hemolysis** separating Strep Species, or the **MacConkey agar** that contains a pH indicator, determining if the organism is a lactose fermenter (fermentation involves the formation of acid, and changes the medium a different color if fermenting). It's okay if these things don't immediately mean anything to you, they will soon.

This next table is for you to start memorizing. Or be a reference tool as you engage organisms to cross-reference.

ORGANISM	AGAR NAME	NOTES ON THE AGAR
Bordetella pertussis	Bordet-Gengou agar	Increased concentration of blood
Legionella	Charcoal yeast extract agar buffered with cysteine and iron	Iron and cysteine in the agar permits growth
Neisseria	Thayer-Martin agar	Selective against everything except *Neisseria*. Vancomycin (staph), TMP, colistin, nystatin
H. influenzae	Chocolate agar with X and V factors	
E. coli, and gram-negative enterics	MacConkey agar	Selective against GPs Lactose fermenters turn the yellow agar **pink**
	Eosin-Methylene Blue agar (EMB)	Selective against GPs Lactose fermenters turn the agar **purple** *E. coli* turns the agar **metallic green**
	Triple Sugar Iron agar (TSI)	**H_2S (black precipitate)** from nonproducers
C. Diphtheriae	Tellurite agar Loffler medium	Black colonies on tellurite

Table 4.1: Agar by Organism
The most important thing here is to see the bug, name the agar, and vice versa (see the agar, name the bug). The notes column is for augmentation. The organism/agar association is must-know. For the enterics, you must know the difference between lactose fermenters and not, and which color they turn the agar.

Pigment-Forming Bacteria

Some bacteria grow colonies that have a particular color. If you are shown the color of the culture growing on an agar, you may be able to skip the vignette altogether, the color being so pathognomonic for the diagnosis.

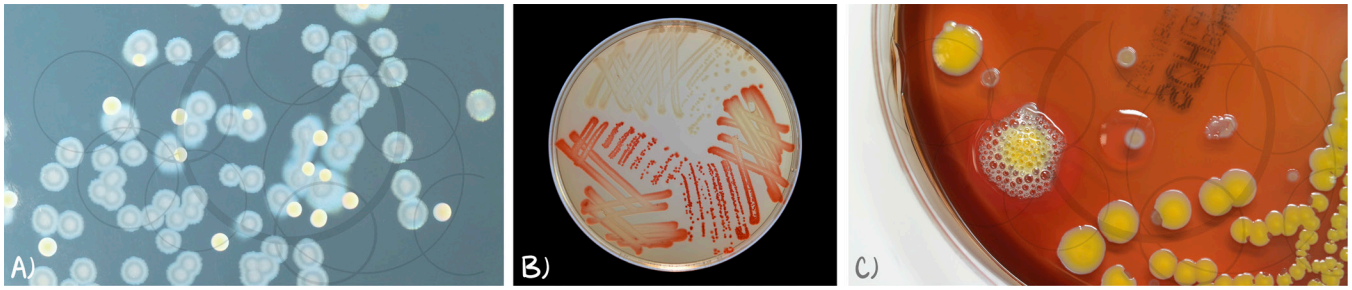


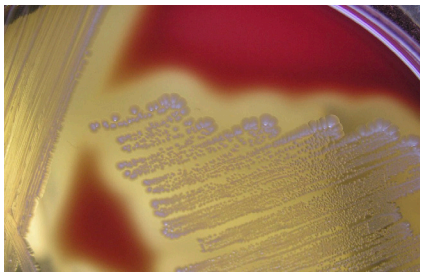
Figure 4.1: Pigmented Colonies
(a) A close-up of *Pseudomonas* colonies that demonstrate a blue-green coloration. Also on this slide are several golden colonies of *Staph. aureus*. (b) This agar bears the red color of *Serratia* colonies on the bottom with nonpigmented colonies on the top. (c) *Staph. aureus* grown on blood agar. The golden yellow colonies are *Staph. aureus*. Also on this agar are grey-to-clear colonies of *Enterococcus*. One golden colony demonstrates a positive catalase test (bubbles), while the grey colony does not.

ORGANISM	PIGMENT	WHY
Actinomyces	Yellow sulfur granules	*N/A*
S. aureus	Yellow pigment	*N/A*
Pseudomonas	Blue-green pigment	*Pyocyanin and pyoverdin*
Serratia	Red pigment	*N/A*

Table 4.2: Pigment Is High Yield
If you get information on pigment, you have the diagnosis. Be careful, because differential media turn yellow and green, so can trip you up. These colors are grown on regular agar; the organisms just happen to produce a pigment.

Hemolysis

Streptococcus is always gram-positive cocci in chains that are catalase negative. That's how you arrive at strep species in the diagnostic workup. You only use hemolysis to differentiate strep species. Other organisms have hemolytic patterns. YOU will not use the hemolytic pattern of any other bacterium to make the diagnosis. This discussion considers only the hemolysis. In the strep lesson, we go over the complete diagnostic pathway and what the hemolysis looks like. While it is often used to decide between species of strep, use caution, *Staph. aureus* can present with β-hemolysis. *Staph. aureus* is catalase positive (not negative like strep), and is in clusters (not chains like strep), so you would not use the hemolytic pattern to determine *Staph. aureus*. It is a data point on the reference sheet, but you are not memorizing that reference sheet.



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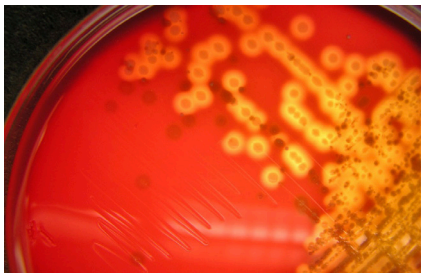

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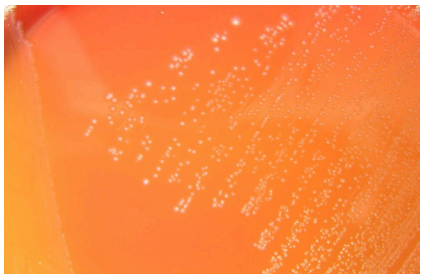


(c)

Figure 4.2: Hemolytic Patterns
(a) A blood agar growing Strep. pyogenes demonstrating complete hemolysis, β-hemolysis. (b) A blood agar growing Strep. pneumoniae demonstrating incomplete hemolysis, α-hemolysis. (c) A blood agar growing Strep. bovis showing no hemolysis, γ-hemolysis.

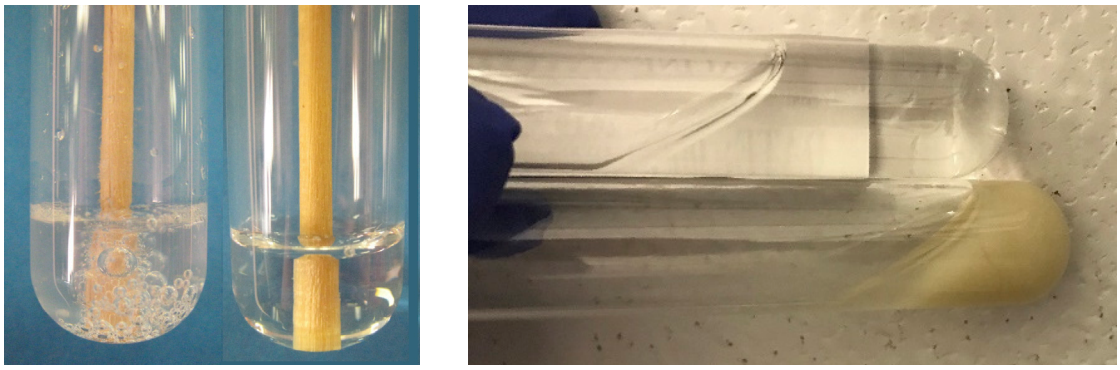
HEMOLYSIS	DESCRIPTION	WHAT TO DO NEXT	POSSIBLE ORGANISM
α-Hemolysis	Some green-brown color, but weak	Optochin sensitivity	Viridans strep *Strep. pneumoniae*
β-Hemolysis	Complete lysis, total clearing of agar	Bacitracin sensitivity (be careful: *Staph. aureus* can perform β-hemolysis)	Group B strep Group A strep
No hemolysis (γ)	No hemolysis, no color change	Growth in 6.5%	*S. bovis* Enterococci

Table 4.3: Hemolysis
Hemolysis is used in identifying gram-positive cocci.

Gram-Positive Specific Tests

Staph is catalase positive. Catalase degrades H_2O_2 into H_2O and O_2. It is a defense mechanism against phagocytosis, neutralizing the chemicals that degrade the bacteria in lysosomes. In the lab, it causes bubbles. Catalase is most important in the determination of the gram-positive cocci. Staph species are catalase positive; strep species are catalase negative. Remember that list of catalase-positive organisms from lesson #1: *Bacterial Structure and Introduction*? See now how it's not useful to memorize that list, since the only way you are going to use that information is to differentiate staph from strep.

Get coagulase only for *Staph. aureus*. Coagulase accelerates the formation of a clot. Coagulase does not coagulation-ase, as the name implies, which would mean that it degrades clots. Coagulase **causes clots**. In a sample in the lab, the bacteria will form a film that adheres to the test tube. **Only *Staph. aureus*** is coagulase positive. All staph species are catalase positive. All strep species are catalase and coagulase negative.



(a) **(b)**

Figure 4.3: Gram-Positive Tests
(a) Catalase test is performed by adding hydrogen peroxide to a sample slide or applying a bacterial swab to a test tube with hydrogen peroxide in it. If there are bubbles, the test is positive. (b) A coagulase test will demonstrate coagulation of the sample. When tilted, a positive test is solid, and does not move. A negative test remains fluid and follows the pull of gravity. Only Staph. aureus is coagulase positive.

Novobiocin is for staph. The coagulase-negative staphs (so not *aureus*) are differentiated by their sensitivity to novobiocin. *Staph. epidermidis* is the contaminant. It's what you want to have on your culture so the person isn't infected. *Staph. epidermidis* lives with us, so is not virulent. It is coagulase negative and sensitive to novobiocin.

Streptococcus is always gram-positive cocci in chains that are catalase negative. That's how you get strep. We use hemolysis to determine the next step. While it narrows the species, it does not define them.

α-Hemolytic streps get optochin sensitivity. Optochin is used to differentiate the α-hemolytic streps. Optochin sensitivity also follows bile solubility. ***Strep. pneumoniae*** is encapsulated. The mechanism of bile salt solubility is not known. Memorize that Strep. pneumoniae is the bug that matters. It is **optochin sensitive** and **soluble in bile**. It also **is encapsulated**. Both optochin and bile kill *Strep. pneumoniae*.

β-Hemolytic streps get bacitracin sensitivity. This is chosen only in the β-hemolytic *Strep.* species. It differentiates GBS from GAS. **Group B strep is resistant**, so it grows. GAS is the other way around. Learn that group B strep is resistant to Bacitracin, so does grow in the presence of Bacitracin, used when there is Beta-hemolysis.

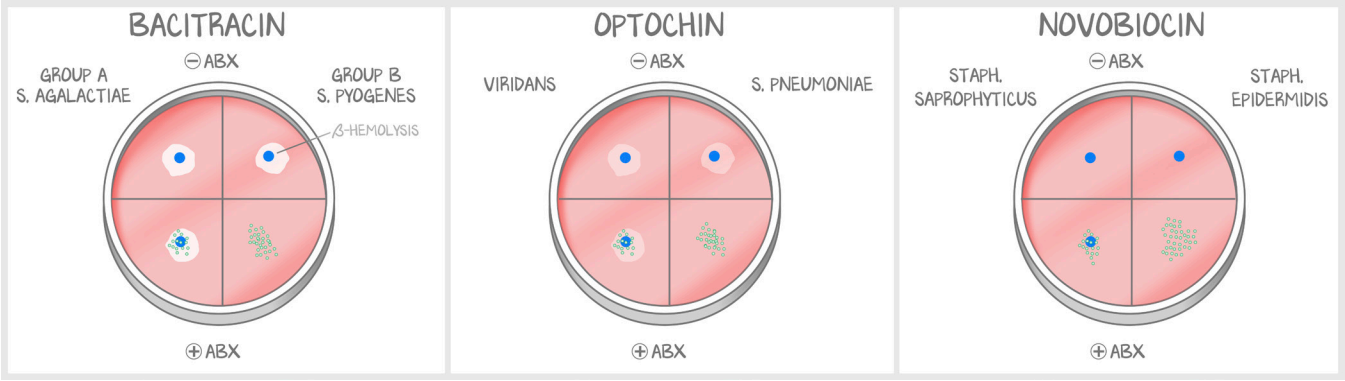


Figure 4.4: Antibiotic Testing
This illustration demonstrates both zones of hemolysis and the concept behind antibiotic testing. For antibiotic testing, the agar plate is divided into quadrants. In one quadrant one organism is plated without antibiotics. In another the same organism is plated with antibiotic added to the agar (illustrated as green speckles). The same thing is performed with another organism in the remaining two quadrants. Bacterial growth is denoted by blue circles. In each instance illustrated, the pattern of hemolysis is insufficient to distinguish the organisms. Only after survival in the face of the antibiotic can the two organisms be differentiated.

TEST	USED ON	POSITIVE	NEGATIVE
Catalase	Gram positives	Staph species have catalase	Strep species do not
Coagulase	Staph species	*Staph. aureus* has coagulase	All other staphs do not
Novobiocin	Coag-negative staph	Novobiocin sensitive is *Staph. epidermidis*	All other staphs are not
Optochin sensitivity	α-hemolytic strep	*Strep. pneumo* is optochin sensitive	Viridans strep is not
Bacitracin	β-hemolytic strep	GBS is resistant	GAS is not

Table 4.4: The Final Strep and Staph Tests

Gram-Positive Algorithm Freebies

TEST	BETWEEN
Catalase	All staph (+) from all strep (-)
Coagulase	*Staph. aureus* (+) from other staph
Novobiocin	*Staph. epidermidis* (sensitive) from *Staph. saprophyticus*
Hemolytic pattern	α-hemolytic strep (partial) β-hemolytic strep (complete) and no hemolysis
Optochin	*Strep. pneumoniae* (sensitive) from viridans (α-hemolytic only)
Bacitracin	Group A strep (sensitive) from group B strep (β-hemolytic only)
Growth in 6.5% NaCl	*E. faecalis* (grows) from *S. bovis* (does not) (γ-hemolytic only)

Table 4.5: Gram-Positive Giveaways

START WITH	DO NEXT	BUGS
Catalase +	Coagulase	Staphs
Coagulase +	Stop	*Staph. aureus*
Coagulase –	Novobiocin	*Staph. epidermidis* dies
Catalase –	Hemolytic pattern	Streps
α-hemolytic pattern	Optochin	*Pneumo* dies, viridans lives
β-hemolytic pattern	Bacitracin	Group A strep dies, group B lives
No hemolytic pattern strep	Growth in 6.5% NaCl or bile	*Enterococcus*

Table 4.6: Next Steps in Diagnostic Algorithm

Gram-Negative Tests

This is going to be nebulous. We have to introduce the tests before we show you how to use them. This is pretty deep in the gram-negative algorithm. They are meaningful tests and we will show you how they end up being selective tests when applied to the appropriate scenario.

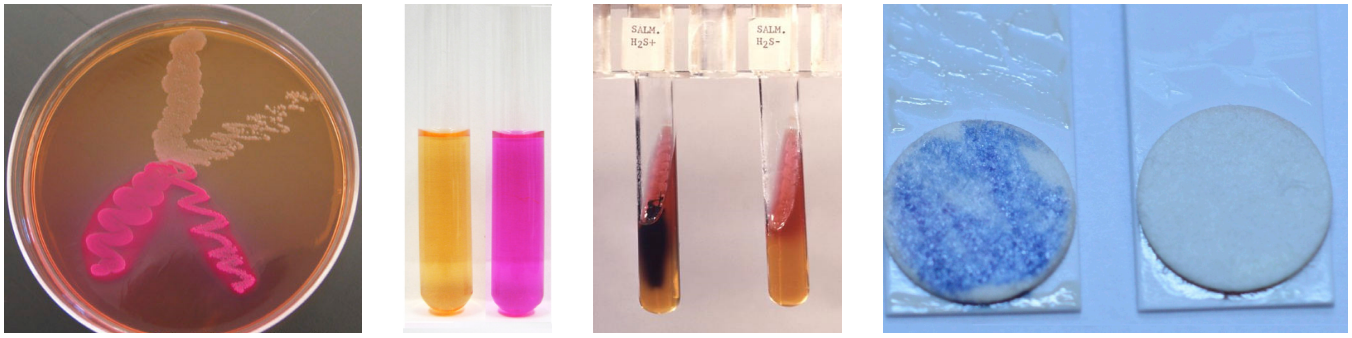
Maltose fermentation is for *Neisseria*. *Neisseria meningitidis*, the meningococci, the really bad meningitis that kills you in 24 hours, is the *Neisseria* that **can ferment maltose**. The other one that causes an STI, *Neisseria gonorrhoeae*, cannot ferment maltose. The Thayer-Martin agar reveals the *Neisseria* genus (it is selective for *Neisseria*) and maltose fermentation reveals the species.

Lactose fermentation is for the enterics, the bugs of the gut flora, the gram-negative rods of the gut, the poop bugs. Those that can ferment lactose will turn the MacConkey agar from normal (yellow) to pink. Lactose fermenters are *E. coli* **and** *Klebsiella*. There are others, such as *Enterobacter*, and then the slow fermenters *Citrobacter* and *Serratia*, but if the early culture report says gram-negative rods that ferment lactose, pick *E. coli* or *Klebsiella*. *Pseudomonas* does not ferment lactose.

Oxidase. Oxidase testing involves taking a sample of the colony and dropping some oxidase reagent on it. It is yellow by default. It turns purple if the organism makes oxidase. The bugs that are oxidase positive are *Pseudomonas* and the comma-shaped rods, *vibrio* and *H. pylori*, as well as *Neisseria* and *Moraxella.* You won't use oxidase to make the diagnosis of any of these organisms, so associate oxidase with *Pseudomonas*.

Urease. Urease cleaves urea. *Proteus*, *Klebsiella*, and *H. pylori* are urease positive.

H_2S production on TSI. The gram-negative rods that are non-lactose fermenters and non-oxidase producers are differentiated by the presence of the ability to make H_2S. The ones that can turn the sample black. The ones that can are *Salmonella* and *Proteus*. All others cannot.



(a) (b) (c) (d)

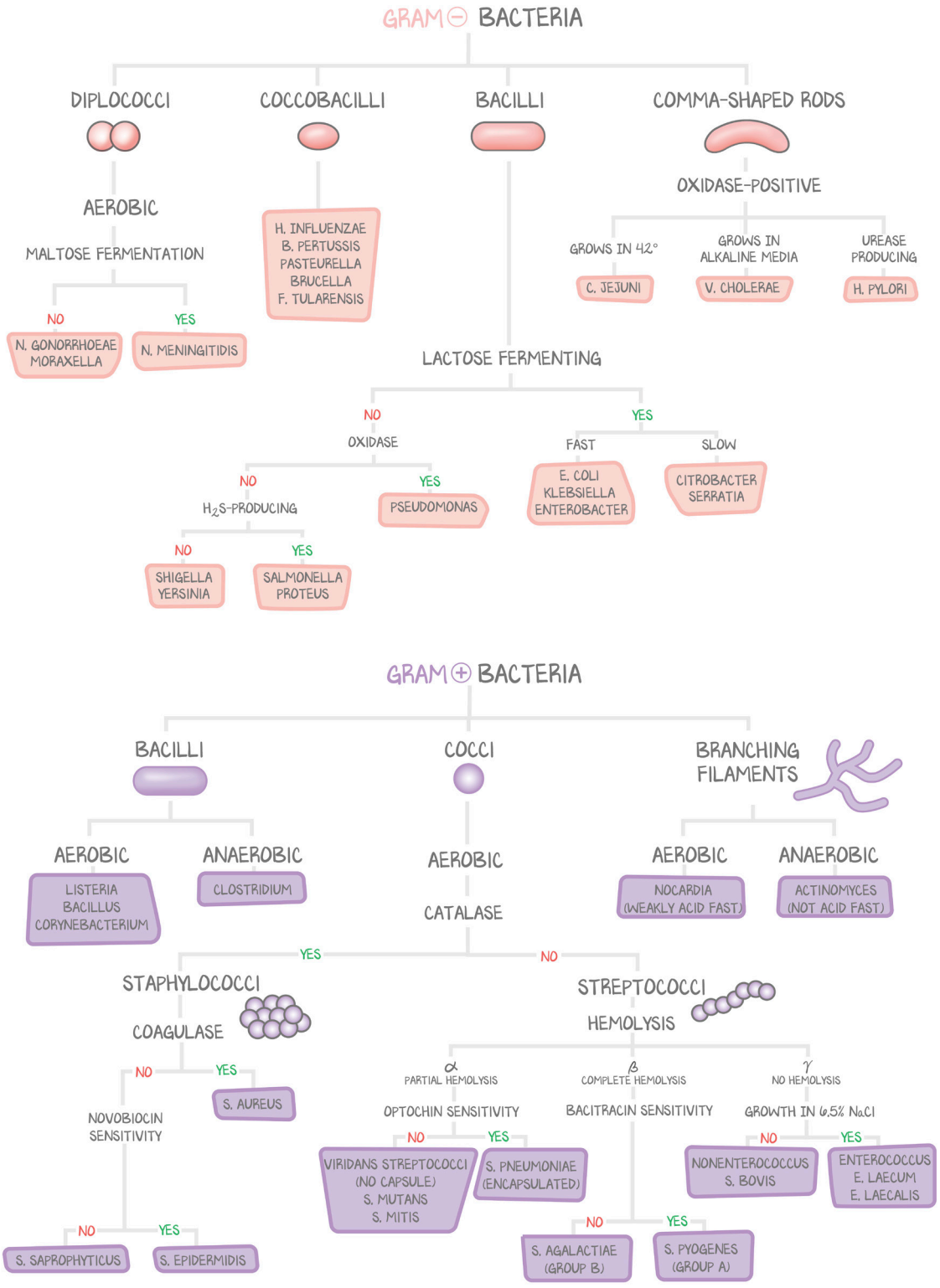
Figure 4.5: Gram-Negative Testing
(a) MacConkey agar starts yellow. It turns red or pink in the presence of acid, indicating lactose fermentation. The colonies themselves will either be red (fermenter) or colorless (nonfermenter). (b) Urease test. If positive, the solution will turn from yellow to pink. (c) Triple Sugar Iron tests for H2S production. If positive, there will be black granules on the agar. (d) Oxidase test turns blue when the organism does produce oxidase. This may be performed on a tab, in a test tube, or by many other means.

FEATURE	WHAT IT MEANS
Maltose fermentation	*N. meningitidis* (+) from *N. gonorrhoeae* (–)
Oxidase positive	Pseudomonas (+) from all other gram negatives (p.s. comma-shaped rods also, and *Neisseria* and *Moraxella*)
H_2S production	*Salmonella* and *Proteus* from all other gram-negative rods
Lactose fermenters	*E. coli* and *Klebsiella* do ferment, separate from all other gram-negative rods (p.s. *Serratia*, *Enterobacter*)
Urease	*Proteus*, *Klebsiella*, *H. pylori*

Table 4.7: Gram-Negative Freebies

The Algorithm for Laboratory Diagnosis

We are providing this because everyone else does. Aside from the above freebies, we really didn't commit this algorithm to memory. There is no rhyme or reason to it. It simply is what is. It is pure memorization. It is unlikely that you will have a pure laboratory diagnosis question with just a panel of tests. You don't get an oxidase test on *Strep.*, and you don't get a hemolytic pattern for *Nocardia*. The information provided should be the proper workup and not curveballs. Do not memorize these algorithms. We do not even recommend starting the algorithm at Gram stain. Our algorithm for teaching this course relies on excluding some organisms to make it work. But what we leave you with works well. This is the laboratory diagnosis that begins with Gram stain.



Conclusion

ZOMFG THERE IS SO MUCH TO KNOW! Yes. There is. This is not how you should go about doing it. This lesson hurt. We'll use what's in it later. It will make sense then. It is out of context here. It is obligatory. The next lesson shows you the OME taxonomy, not based on laboratory algorithms, organized on how you will use microbiology, not how microbiologists in a lab runs their tests (which was this lesson).